**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants : Nilsson

Serial no. : 08/981,098

Filed : 17 December 1997

For : Metabolically engineered lactic acid bacteria and their use

Examiner : Afremova, V

Art unit : 1651

**Declaration of Claus Maxel Henriksen**

1. I, **Claus Maxel Henriksen**, employed by Chr. Hansen A/S, do state and declare as follows:

2. I believe that I am a person skilled in the art to which the above-captioned application pertains. Please find attached to this declaration my Curricula Vitae.

3. I have read and understood the pending claims in that application as well as the Office Action related thereto dated 19 December 2000, and in respect to said Office Action I have the following comments:

4. I have made a detailed comparison of the metabolic pathways of pyruvate catabolism in *Escherichia coli* relative to lactic acid bacteria in order to show that there are a number of important and distinctive differences between these two bacteria.

5. Introduction

Bacterial fermentation is a condition under which the growth of the bacterium occurs without any exogenous electron acceptor. The metabolism of the gram-positive lactic acid bacterium *Lactococcus lactis*, and many other lactic acid bacteria, is exclusively fermentative, whereas *e.g.* the gram-negative enteric bacterium *E. coli* is capable of both fermentative and respiratory growth. Although the two bacteria share some fea-

tures with respect to their fermentative metabolism, a number of important and distinct differences exist.

As soon as oxygen (or an alternative electron acceptor) becomes available for *E. coli*, a shift from fermentative to respiratory metabolism takes place. Under such conditions, the metabolic and physiological states of the two bacteria are no longer comparable.

In the following paragraphs the fermentative and respiratory metabolic pathways of *L. lactis* and *E. coli* are described in further details. *L. lactis* was taken as representative for the group of lactic acid bacteria since this microorganism is the best characterised microorganism among the lactic acid bacteria. Emphasis is put on the intracellular redox balance with respect to NADH and $NAD^+$ since this balance is a major determinant of whether growth takes place or not. The maintenance of the redox balance furthermore influences the pattern of fermentation products.

6. Fermentative metabolism of *Lactococcus lactis*

As mentioned above, the metabolism of *L. lactis* is exclusively fermentative. The bacterium does not possess an active tricarboxylic acid cycle (Krebs cycle) nor porphyrins and cytochromes. The generation of metabolic energy, in the form of ATP, therefore relies on substrate-level phosphorylation.

When a wildtype strain of *L. lactis* is grown under anaerobic conditions on a carbon source, *e.g.* glucose or lactose (if the genetic determinants for lactose metabolism are present), a large majority of the carbon source is converted into L-(+)-lactic acid via the intermediate metabolite pyruvate (Fig. 1). Under such conditions, the metabolism is refered to as homolactic. The series of enzymatic reactions which convert glucose into pyruvate constitutes the glycolytic pathway. Other sugars than glucose enter the glycolytic pathway at various stages or is converted into pyruvate by alternative pathways. Pyruvate is therefore situated at a very important and central point in the sugar catabolism. As it can be seen from Fig. 1, the conversion of glucose into L-(+)-lactic acid generates two molecules of ATP but is neutral with respect to the redox equivalents NADH and $NAD^+$. During homolactic fermentation, other metabolic end-products are only formed in small amounts.

Deviations from homolactic fermentation take place i) for growth on carbon sources metabolised slowly, ii) under aerobic conditions, or iii) if the strains carries one or more enzymatic defects in the fermentative pathway (defects elsewhere in the metabolism might ultimately also affect the fermentation). In these cases other metabolites than L-(+)-lactic acid are formed in significant amounts, *e.g.* formic acid, acetic acid, acetaldehyde, ethanol, acetoin, α-acetolactic acid, diacetyl, 2,3-butanediol and carbon dioxide. It is, however, important to note, that the consumption and generation of the redox equivalents NADH and $NAD^+$ are still balanced. In case where oxygen is present, the enzyme NADH oxidase helps in maintaining the redox balance as depicted on Fig. 1.

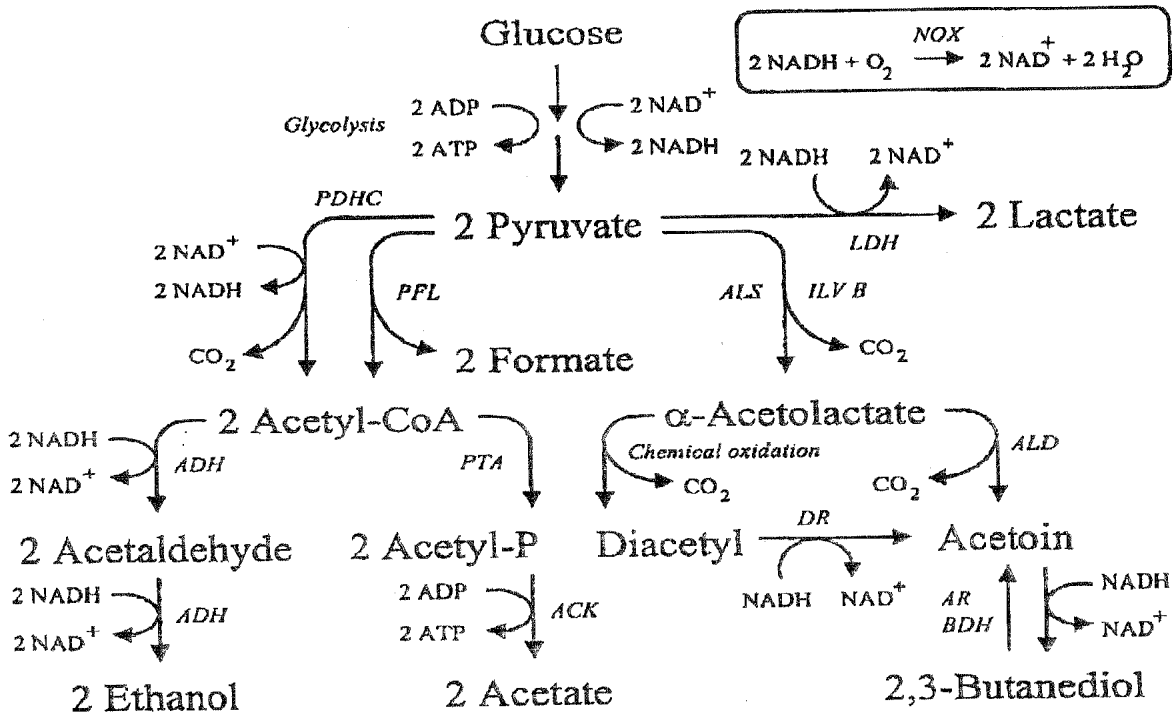


**Fig. 1.** The pyruvate metabolism of *L. lactis*. Abbreviations for enzymes: LDH: L-(+)-Lactate dehydrogenase; PDHC: Pyruvate dehydrogenase complex; PFL: Pyruvate formate-lyase; ADH: Acetaldehyde and alcohol dehydrogenase; PTA: Phosphotransacetylase; ACK: Acetate kinase; ALS / ILV B: Catabolic and anabolic α-acetolactate synthase; ALD: α-acetolactate decarboxylase; DR: Diacetyl reductase; AR: Acetoin reductase; BDH: Butanediol dehydrogenase; and NOX: NADH oxidase.

Another important intermediate metabolite is acetyl-CoA which is an essential precursor in biosynthetic reactions, *e.g.* lipid biosynthesis. Under anaerobic conditions, acetyl-CoA is formed from pyruvate by pyruvate formate-lyase. However, this en-

zyme becomes irreversibly inactivated under aerobic conditions (Takahashi *et al.*, 1982; 1987). Instead, acetyl-CoA is formed from pyruvate by the pyruvate dehydrogenase complex. In *L. lactis*, this $NAD^+$ dependent enzyme complex is only active under aerobic conditions (Snoep *et al.*, 1993) and requires lipoic acid (and co-enzyme A and thiamine) to be functional. However, *L. lactis* can in contrast to *E. coli* not synthesise lipoic acid *de novo* since no genetic determinants are present in the genomic sequence (Bolotin *et al.*, 1999), and lipoic acid therefore needs to be present in the medium for the activity of the enzyme complex (Cogan *et al.*, 1989; Collins and Bruhn, 1970; Reed *et al.*, 1951; Reiter and Oram, 1962). In case acetyl-CoA cannot be formed by either the activity of pyruvate formate-lyase or the pyruvate dehydrogenase complex, the supply of exogenous acetate becomes a prerequisite for growth.

7. Fermentative metabolism of *Escherichia coli*

*E. coli* and *L. lactis* share some features with respect to the fermentative metabolism. However, the fermentative metabolism of *E. coli* differs from the fermentative metabolism of *L. lactis* on some important characteristics. Whereas the anaerobic fermentation of *L. lactis* is almost exclusively homolactic with L-(+)-lactic acid as end-product, the fermentation products of *E. coli* comprise a mixture of ethanol, acetic acid, formic acid (partly cleaved to hydrogen and carbon dioxide by the enzyme formate hydrogen-lyase), D-(-)-lactic acid and succinic acid (Böck and Sawers, 1996). As with the metabolism of *L. lactis*, maintenance of the redox balance in the fermentative pathway of *E. coli* is required for growth to proceed.

The capacity to produce α-acetolactic acid, acetoin, diacetyl and 2,3-butanediol is widely distributed among microorganisms. The production of acetoin is the basis of one of the most frequently used identification tests in bacteriology, the Voges-Proskauer reaction. In contrast to *L. lactis*, *E. coli* is not capable of producing α-acetolactic acid, acetoin, diacetyl or 2,3-butanediol (Böck and Sawers, 1996).

Yet another difference between *L. lactis* and *E. coli* is that the latter microorganism is oxidase negative (Neidhardt, 1996), whereas the former microorganism expresses *e.g.* the NADH oxidase described in the previous section.

8. Respiratory metabolism of *Escherichia coli*

*E. coli* is capable of performing respiration with a variety of chemical compounds as electron acceptors, e.g. fumarate, nitrate, nitrite, oxygen, *S*- and *N*-oxide compounds, tetrathionate and thiosulfate (Gennis and Stewart, 1996). The respiratory pathway described in most details is the aerobic respiration with oxygen as electron acceptor.

In the case of full respiratory metabolism with oxygen as electron acceptor, *E. coli* catabolises glucose into carbon dioxide and water via glycolysis, TCA cycle (Cronan and LaPorte, 1996) and an electron transport chain. Along the catabolism, metabolic energy in the form of ATP is generated (both via substrate-level phosphorylation and oxidative phosphorylation), and this energy is withdrawn together with a number of precursor metabolites for biosynthetic purposes.

The glycolysis and the TCA cycle are linked together by the pyruvate dehydrogenase complex which converts pyruvate into acetyl-CoA. As mentioned above, lipoic acid is an essential part of the enzyme complex. In contrast to *L. lactis*, *E. coli* is fully capable of synthesising and incorporating lipoic acid in the enzyme complex. In the TCA cycle, acetyl-CoA is catabolised into carbon dioxide with a concomitant generation of ATP (and GTP) and reduced redox equivalents NADH (and $FADH_2$). The reduced redox equivalents are subsequently oxidised via the electron transport chain where the electrons are donated to oxygen which becomes reduced to water. Along the electron transport chain, protons are pumped across the inner plasma membrane thereby establishing a proton motive force across the membrane. This proton motive force is subsequently used for driving ATP synthesis (oxidative phosphoryiation) by the membrane-bound ATP synthase complex ($F_oF_1$-ATPase).

9. Mutants of *L. lactis* and *E. coli* with defective PFL and LDH

The isolation and characterisation of a PFL and LDH defective mutant of *E. coli* has previously been reported (Mat-Jan *et al.*, 1989). According to the report, such a double mutant strain is not capable of anaerobic growth even in the presence of acetate unless conditions for anaerobic respiration with nitrate or fumarate as electron acceptors are provided. Under aerobic conditions, the strain displayed growth even in the absence of acetate.

The lack of fermentative growth of the double mutant reflects the problems associated with the maintenance of the intracellular redox balance. The reduced redox

equivalents generated via glycolysis can no longer be re-oxidised in the remaining part of the fermentative metabolism due to the two enzymatic defects. The redox balance and thereby growth are only restored under respiratory conditions with either nitrate, fumarate or oxygen as electron acceptor.

A similar double mutant strain of *L. lactis* was found to be strictly aerobic. Furthermore, the aerobic growth of this strain required supply of exogenous acetate. While a double mutant of *E. coli* depends on respiratory metabolism (with either nitrate, fumarate or oxygen as electron acceptor) for growth, the growth of the double mutant of *L. lactis* depends on a combined action of the NADH oxidase (and thereby aerobic conditions) and the pathway leading to the formation of acetoin, α-acetolactic acid, diacetyl and 2,3-butanediol.

The fact that a PFL/LDH double mutant strain of *E. coli* could be isolated according to Mat-Jan *et al.* (1989) can therefore not be taken as an indication that it was to be expected that a corresponding double mutant of *L. lactis* would also have the ability to grow and produce metabolites under aerobic conditions. Since the growth of the double mutant strain of *E. coli* depends on metabolic pathways which are not present in *L. lactis* (and vice versa), a successful isolation of a double mutant strain of the latter microorganism could therefore not be foreseen – even with knowledge of the report of Mat-Jan *et al.* (1989). In case the combined action of the NADH oxidase and the pathway leading to the formation of acetoin, α-acetolactic acid, diacetyl and 2,3-butanediol could not fully compensate the lack of NADH re-oxidation by LDH, a double mutation would have been lethal for *L. lactis*. The fact that the isolation procedures applied for obtaining the double mutant strains of *E. coli* and *L. lactis* are essentially the same, a successful isolation of a double mutant strain of the latter microorganism could not have been foreseen since the two microorganisms are so distinct with respect to their aerobic metabolism, as described above in much details.

## 10. References

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10. I further declare that all statements made herein of my own knowledge are true, and further that the statements were made with the knowledge that wilful false state-

ments and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon.

Dated: June 7, 2001 Signature: _____

Claus Maxel Henriksen

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## Chemical Engineer, PhD.

### Personal details


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### Education and employment

| | |
|---|---|
| 2000-2001 | Section Manager Microbial Metabolism, Chr. Hansen A/S, Hørsholm.<br>Manager for three chemists and four technicians.<br>Microbial physiology. Optimisation of fermentations conditions. Metabolic analysis. Enzyme purification. Initiation of high-throughput screening laboratory. |
| 1996-2000 | Chemist, Department of Physiology & Metabolism, Chr. Hansen A/S, Hørsholm.<br>Isolation and characterisation of mutant strains of various lactic acid bacteria. Fermentation optimisation in laboratory and pilot scale.<br>Participation in a number of patent applications. |
| 1993-1996 | Ph.D., Department of Biotechnology, Technical University of Denmark.<br>Supervisors: Prof. Jens Nielsen and Prof. John Villadsen.<br>In collaboration with Novo Nordisk A/S and later Royal Gist-Brocades N.V.<br>Metabolic Characterisation of *Penicillium chrysogenum*:<br>Microbial physiology of a high-yielding strain of *P. chrysogenum* during continuous cultivations in high-performance bioreactors. Quantitative study of growth and product formation. Metabolic flux analysis. Characterisation of the enzyme ACV synthetase. |
| 1993 | "Studentermedhjælp" in pilot plant, Novo Nordisk A/S, Bagsværd. |
| 1988-1993 | M.Sc., Department of Biotechnology, Technical University of Denmark.<br>Metabolic Flux Analysis of *Penicillium chrysogenum*.<br>Continuous Cultivation of *Penicillium chrysogenum*. |
| 1988-1993 | Part time job at Hamatini A/S, Lyngby (weekends and evenings). |
| 1985-1988 | Student at Sct. Knuds Gymnasium, Odense. |

## Courses

Modelling and on-line monitoring of fermentation processes. Center for Process Biotechnology, Lyngby, Denmark. June 1st-11th, 1993.

Advanced Course on Microbial Physiology and Fermentation Technology. Delft University of Technology, Delft, The Netherlands. December 9th-20th, 1996.

Chr. Hansen Academy, Professionals Programme. Internal course at Chr. Hansen A/S for specialists. November 25th-27th, 1997 and January 27th-29th, 1998.

"Økonomi for ikke-økonomer". Arranged by IBC Euroforum ApS. Copenhagen., November 15th-16th, 2000.

Chr. Hansen Academy, Good Leadership. Internal course at Chr. Hansen A/S. January 22nd-26th, 2001.

Chr. Hansen Academy, Good Employeeship. Internal course at Chr. Hansen A/S. Marts 26th-27th, 2001.

"Coaching". Arranged by IBC Euroforum ApS. Copenhagen., May 9th-10th, 2001.

## Languages

Danish: Mother tongue.
English: Fluent, spoken as well as in writing.
German: Spoken and read.
Others: Knowledge in French and Spanish.

## Other personal skills

Driving licence.

Skilled user of personal computers incl. the use of Microsoft Office Package, Microsoft Internet Explorer, Lotus SmartSuite, Lotus Notes, Matlab and a number of applications for control and data acquisition of fermentation processes.

## Activities and interests

French culture incl. travelling in France, especially in the southern part.
Watching films and visiting art galleries.

## Publications

**Christensen, L. H., Henriksen, C. M., Nielsen, J., Villadsen, J. & Egel-Mitani, M. (1995).** Continuous cultivation of *Penicillium chrysogenum*. Growth on glucose and penicillin production. *J. Biotechnol.*, **42**, 95-107.

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**Henriksen, C. M., Nielsen, J. & Villadsen, J. (1997).** Influence of the dissolved oxygen concentration on the penicillin biosynthetic pathway in steady-state cultures of *Penicillium chrysogenum*. *Biotechnol. Prog.*, **13**, 776-782.
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**Curic, M., de Richelieu, M., Henriksen, C. M., Jochumsen, K. V., Villadsen, J. & Nilsson, D. (1999).** Glucose/citrate cometabolism in *Lactococcus lactis* subsp. *lactis* biovar. diacetylactis with impaired α-acetolactate decarboxylase. *Metabolic Eng.*, **1**, 291-298.
**Henriksen, C. M., Nilsson, D., Hansen, S. & Johansen, E. (1999).** Industrial applications of genetically modified microorganisms: gene technology at Chr. Hansen A/S. *Int. Dairy J.*, **9**, 17-23.
**Høier, E., Janzen, T., Henriksen, C. M., Rattray, F., Brockmann, E. & Johansen, E. (1999).** The production, application and action of lactic cheese starter cultures. *In* Technology of Cheesemaking (ed. B. A. Law), Sheffield Academic Press Ltd., Sheffield, pp. 99-131.
**Walfridsson, M., Henriksen, C. M. & Nilsson, D. (1999).** Novel genetically modified lactic acid bacteria having modified diacetyl reductase activities. PCT patent application WO 99/54453.
**Henriksen, C. M., Curic, M. & Nilsson, D. (2000).** Citrate can partially replace carbon dioxide required for growth of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. *Lett. Appl. Microbiol.*, **30**, 415-418.
**Henriksen, C. M. & Nilsson, D. (2000).** Redirection of pyruvate catabolism in *Lactococcus lactis* by selection of mutants with additional growth requirements. *Appl. Microbiol. Biotechnol.*, accepted.

## Posters and presentations

**Henriksen, C. M., Nielsen, J. & Villadsen, J. (1995).** Influence of phenoxyacetic acid on growth and product formation of a penicillin V producing strain of *Penicillium chrysogenum*. Poster presented at the 7th European Congress on Biotechnology, Nice, February 19-23.
**Henriksen, C. M., Nielsen, J. & Villadsen, J. (1995).** Continuous cultivation of *Penicillium chrysogenum*: Influence of phenoxyacetic acid on growth and product formation. Poster presented at the Danish Biotechnology Conference. Enzymes - Design and Production, Vejle, May 18-19.
**Theilgaard, H. B. Aa., Henriksen, C. M. & Nielsen, J. (1995).** ACV synthetase of *Penicillium chrysogenum*. Poster presented at the Danish Biotechnology Conference. Enzymes - Design and Production, Vejle, May 18-19.
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**Theilgaard, H. B. Aa., Henriksen, C. M. & Nielsen, J. (1998).** Purification and characterization of ACVS from *Penicillium chrysogenum*. Poster presented at the 4th European Conference on Fungal Genetics, León, April 2-8.
**Henriksen, C. M. & Nilsson, D. (1998).** Metabolic redirection of pyruvate in *Lactococcus lactis*. Poster presented at the 25th International Dairy Congress, Aarhus, September 21-24.
**Henriksen, C. M. & Nilsson, D. (1999).** Redirecting pyruvate catabolism in *Lactococcus lactis*. Poster presented at the 6th Symposium on Lactic Acid Bacteria, Veldhoven, September 19-23.